pCAR and its uses

The invention provides improvements in the field of animal models for testing effects of genes introduced into animal cells or tissue by adenoviral gene transfer.

Adenoviruses infect cells using two cell surface receptors, the "Coxsackie B and adenovirus 2 and 5 receptor" (hereinafter referred to as CAR; Bergelson J.M., et al, Science 275, 1320-23, 1997) and the integrin receptors ($\alpha v\beta 3$ or $\alpha v\beta 5$; Wickham, T.J. et al, Cell 73, 309-19, 1993) the contents thereof being incorporated herein by reference. Adenoviral based vectors are widely used in gene therapy, as they represent one of the most efficient ways to deliver genes to target cells. They are of particular interest for in vivo gene therapy proof-of concept experiments in rodent models. However, rodent tissues are not well transducible with adenoviral vectors.

In its broad aspect the invention is concerned with genetic modification of target cells which are normally refractory to adenoviral transduction. More particularly the invention provides a plasmid construct that expresses a porcine adenovirus receptor (pCAR) and transgenic animals that show expression of pCAR.

Organ transplants of liver, kidney, lung and heart are now regularly performed as treatment for endstage organ disease. Despite the use of modern immunosuppressive drugs acute and chronic graft (tissue or organ) rejection still remain major factors in graft loss. There is, therefore, a continued need for means to inhibit acute and chronic graft rejection and increase graft acceptance, e.g. through induction of peripheral tolerance without causing serious toxic side effects typically associated with conventional immunosuppressant therapy. When considering cell transplantation, e.g. bone marrow derived cells, islet cells, neuronal cells etc. one is faced with similar problems of rejection. Making organs or cells less immunogenic through genetic modification is seen as an alternative or add on to conventional immunosuppression.

Rodent animal models are of crucial importance for testing the immunomodulatory effects of new gene products. However in the case of using adenovirus as gene delivery vehicle rodent models have so far proven to be of limited value, as many rodent organs or cell

types are refractory to adenoviral transduction. This may be due to the fact that either the adenoviral receptor CAR is not expressed or only weakly expressed on the cell surface of the cells of interest.

Accordingly, the invention provides a plasmid or vector construct that comprises a DNA molecule which expresses porcine CAR (SEQ ID NO:4ereinafter referred to as pCAR) or a biologically active fragment or derivative thereof, for example a C-terminally truncated porcine CAR (SEQ ID NO:2hereinafter referred to as ΔpCAR), that retains full functionality as adenoviral receptor.

pCAR comprises an intracellular domain, a transmembrane domain and a an extracellular domain that binds to the adenoviral fibre proteins, i.e. a total sequence of 365 amino-acids. It will be understood that any nucleic acid sequence encoding a porcine CAR homologue is a candidate for utilization in the present invention. For example, it may include a pCAR sequence with a modified, mutated or truncated region thereof, that retains the activity of mediating adenoviral transduction. It will be further understood by the skilled person that any nucleic acid sequence which encodes a biologically active form of pCAR, including but not limited to a genomic or cDNA sequence or functionally equivalent variant or mutant thereof or a fragment thereof which encodes a biologically active protein fragment or derivative which mediates adenoviral transduction, may be utilized in the present invention. For example, ΔpCAR may comprise the leader sequence of 19 amino-acids, the extracellular domain of 216 amino-acids, the transmembrane domain of 24 amino-acids and a truncated cytoplasmic domain, e.g. limited to 3 amino-acids. Two potential sites for N-glycosylation are located at Asn 106 and Asn 201. Amino-acids present in the sequence which are not essential to the activity may be changed by mutation, e.g. amino-acid 258 may be changed from Val to Ile; amino-acid 262 may be changed from His to Arg.

Preferred nucleic acid sequence for use in the invention is e.g. as disclosed in SEQ ID NO: 1 from nucleotide 3229 to nucleotide 4014. The corresponding amino acid sequence encoded by such DNA sequence is indicated in SEQ ID NO:2.

Any known expression vector or plasmid that is capable of expression upon transfection of a specified eukaryotic target cell may be utilized to pratice the invention. "Plasmid" and "vector" can be used interchangeably in the present specification as the plasmid is the most

commonly used form of vector. An expression vector is a vector capable of directing the expression of genes to which they are operatively linked. An operable linkage as used herein refers to the position, orientation and linkage between a structural gene and expression control element(s) such that the structural gene can be expressed in any host cell. The term "expression control element" includes promoters, enhancers, ribosome binding sites etc. Any eukaryotic promoter and/or enhancer sequences available to the skilled person which are known to control expression of the nucleic acid of interest may be used in plasmid vector constructs, including but not limited to a cytomegalovirus (CMV) promoter, a Rous Sarcoma (RVS) promoter, a Murine Leukemia (MLV) promoter, a herpes simplex virus (HVS) promoter, such as HSV-tk, a β-actin promoter, e.g. chicken β-actin, as well as any additional tissue specific or signal specific regulatory sequence that induces expression in the target cell or tissue of interest. A preferred expression vector or plasmid according to the invention is e.g. an eukaryotic expression vectors, e.g. a pβ-actin-p16PL vector such as p(chicken)β-actin-p16PL.

In one such embodiment, a DNA sequence encoding pCAR is subcloned into the DNA plasmid expression vector, e.g. pβ-actin-p16PL, resulting in pβ-actin-pCAR-p16PL. p16PL is a standard mammalian expression vector, containing a gene that encodes a selectable marker, e.g. an antibiotic resistance gene, and a β-actin promoter active in mammalian cells (K. M. Marsden et al, J. Neurosc., May 15, 1996, 16(10): 3265-3273). Such a construct, which may be constructed by one of ordinary skill with components available from numerous sources, will drive expression of a pCAR DNA fragment ligated downstream of the β-actin promoter subsequent to transfection of the target cell. More specifically, pCAR is cloned from pig liver RNA using a PCR based approach. The PCR fragment is inserted into the expression vector pSport (Life Technologies). This plasmid serves as template to create the truncated version of ΔpCAR. Preferably pβ–actin is pβ-(chicken) actin.

The invention further provides host cells into which a recombinant expression vector of the invention has been introduced. A host cell can be any prokaryotic or eukaryotic cell, e.g. bacterial such as E. Coli, yeast or mammalian cells, e.g. CHO or COS cells.

The host cells of the invention may preferably be used to produce nonhuman transgenic animals, preferably a mammal, more preferably a rodent such as a rat or mouse, or a pig.

For example, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which a pCAR-coding sequence has been introduced. A transgenic animal of the invention, more preferably a mammal, most preferably a rodent or a pig, may be created by introducing a pCAR expression construct into the male pronuclei of a fertilized oocyte, e.g. by microinjection, or into embryonic stem cells, e.g. by electroporation. Methods for generating transgenic rodents have become conventional in the art and are described e.g. in USP 4,736,866, 4,870,009, 4,873,191, or in Manipulating the Mouse Embryo, B. Hogan, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). For example the expression construct may be introduced into an embryonic stem cell line and cells in which the introduced pCAR gene has integrated are selected. The selected cells are then used to produce chimaeras with known standard procedures. A chimaeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. The pCAR expression plasmid may also be inserted into somatic/body cells of the donor animal to provide a somatic recombinant animal, from whom the DNA construct is not capable of being passed on to offspring. For example, a somatic cell from the transgenic animal can be isolated and induced to exit the growth cycle and enter G_o phase. The quiescent cell can then be fused, e.g. through the use of electrical pulses, to an enucluated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyst and then transferred to pseudopregnant female foster animal. The offspring of this female foster animal will be a clone of the animal from which the somatic cell is isolated. Or for example, an improved method of cloning pigs using donor nuclei from non-quiescent differentiated cells in which the desired DNA, e.g. porcine CAR or fragments or variants thereof, is inserted in said differentiated pig cell or pig cell nucleus. This improved method is described in US 6,235,969 B1 and is hereby incorporated by reference.

The present invention also provides a method for improving adenoviral gene transfer in a rodent using a transgenic rodent which expresses or overexpresses pCAR. Such rodents may be used as models in gene therapy to test adenoviral transduction, e.g. prevention or treatment of acute or chronic graft rejection, autoimmune disorders, e.g rheumatoid arthritis, cardiovascular disorders, e.g. restenosis, nervous system disorders, e.g. parkinson disease, etc. A preferred embodiment of the invention is the use of such rodents expressing or overexpressing pCAR in transplantation experiments, for example, of organs, tissues or cells, e.g. lung, heart, kidney, liver, pancreas, small bowel, spleen, pancreatic islets,

neuronal or stem cells, etc. For example, organs, tissues or cells of such transgenic rodents, e.g. mice, are removed, in vitro transduced with the adenoviral gene delivery vector to be tested and then transplanted into rodents, e.g. mice, e.g. such animals which do not express pCAR.

The functional expression of pCAR, e.g. ΔpCAR may also be used to generate transgenic pigs that overexpress this adenoviral receptor. Porcine organs, tissues or cells transgenically modified to express high levels of pCAR may be used as recipients for adenoviral gene therapy vectors. Such transgenic modified organs, tissues or cells can be transfected with adenoviral gene therapy vectors carrying thrapeutically beneficial genes either ex vivo or in vivo and can be subsequently transplanted in a recipient. Beneficial genes are those that are expected to confer graft protection following transplantation of these gene delivered organs in xenotransplantation therapy. The present invention comprises a method to generate such transgenic pigs expressing high levels of pCAR or a functionally equivalent variant or mutant thereof or a fragment thereof, e.g. as disclosed above, and gene therapy methods for preventing or inhibiting graft rejection in a recipient using organs, tissues or cells of such transgenic pigs.

Definitions

To facilitate understanding of the invention, a number of terms are defined below.

"Fragment" of a polypeptide sequence refers to a polypeptide sequence that is shorter than the reference sequence but that retains essentially the same biological function or activity as the reference polypeptide. "Fragment" of a polynucleotide sequence refers to a polynucloetide sequence that is shorter than the reference sequence of SEQ ID NO: 2 and 4.

"Transduction" Transfer of genetic material or characteristics from one bacterial cell to another by the incorporation of bacterial DNA into a bacteriophage.

"Variant" refers to a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide, but retains the essential properties thereof. A typical variant of a polynucleotide differs in nucleotide sequence from the reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid

sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from the reference polypeptide. Generally, alterations are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, insertions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. Typical conservative substitutions include Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln-I Ser, Thr; Lys, Arg; and Phe and Tyr. A variant of a polynucleotide or polypeptide may be naturally occurring such as an allele, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis. Also included as variants are polypeptides having one or more post-translational modifications, for instance glycosylation, phosphorylation, methylation, ADP ribosylation and the like. Embodiments include methylation of the N-terminal amino acid, phosphorylations of serines and threonines and modification of C- terminal glycines.

The following Exampes are illustrative only and not limiting of the invention. The β-actin promotor used in the Examples is the β-(chicken)actin promotor.

Example 1: Construction of the expression vector

The full length cDNA for porcine CAR is cloned from pig liver using degenerated primers (forward: 5'-accatggcgckcctrctgt-3' and reverse: 5'-catatggaggctytatacya-3' in which k=g or t; r=a or g and y=c or t). The PCR fragment is bluntend inserted into the vector pSport (Life Technologies). Porcine CAR (SEQ ID NO:4) has an overall aminoacid homology of 91% to human as well as mouse CAR. This clone is used as template to generate the ΔpCAR gene as disclosed in SEQ ID NO:1 from nucleotide 3229 to nucleotide 4014, using PCR. The primers used to generate this construct contain two amino acid changes at the C-terminal end of the construct. The sense primer SpeI-CAR (5'–ggactagtgccaccatggcgctcctgctgtgcttc–3') is located at position 1-21 of pCAR and contains a SpeI site, a Kozak sequence and the start codon. The antisense primer CAR-XbaI (5'–gctctagattaacgacagcaaaagatgataagacc–3') is located at position 760-786 of porcine CAR containing a stop codon and a XbaI site. The

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PCR amplification used the following conditions: 1x native *Pfu* buffer, 2.5mM $MgCl_2$, 0.2mM dNTPs, 2.5 U native *Pfu* polymerase (Stratagene) and 20pmol SpeI-CAR and CAR-XbaI (each). Porcine CAR cDNA (5ng) is used as template and hot start PCR is performed using the following profile: 1x (5min 95°C) 20x (30sec 95°C, 1min 55°C, 1min 30sec 72°C) 1x (3min 72°C). A PCR product of a predicted size of 788bps is obtained and separated on a 1% low melting agarose gel (SeaPlaque GTG; FMC). The band is excised and the PCR product isolated from the gel piece using the QIAquick gel extraction kit from Qiagen according to the manufacturers protocol. The isolated PCR product is then digest with *XbaI* (LifeTechnologies) and repurified as described above. The digested purified PCR product is ligated into *MscI-XbaI* digested pβactin-16PL vector.

INVaF'chemically ultracompetent bacteria from Invitrogen are transformed and 48 colonies picked, rescreened by PCR using SpeI-CAR and CAR-XbaI as primers. From 48 colonies analyzed 20 contain the insert – 12 are selected for DNA sequencing. The sequencing primer actinsense (5'-accggcggggtttatatcttc–3') is the 5'-primer located just upstream of the MCS of the pβactin-16PL vector. Actinanti (5'-cctctacagatgtgatatggc–3') is the 3'-primer located just downstream of the MCS of pβactin-16PL vector. The nucleotide sequence of the β-actin promotor, the ΔpCAR gene and the SV40 polyadenylation signal is shown in SEQ ID NO:1.

Example 2: In vitro expression of ΔpCAR in mammalian cells (Western blot)

A human lung carcinoma cell (A30), rat embryonic fibroblasts (Rat2, ATCC:CRL-1764) and chinese hamster ovary cells (CHO) are used for transient transfections. Culture conditions are as follows:

Cell Line	Medium	Serum	Supplement	Antibiotics
A30	RPMI	10%FBS	1%NEAA	1%PS
Rat2	DMEM	10%FBS		1%PS
CHO	αMEM	10%FBS		1%PS

In addition, all media contain 2mM Glutamax II. Cultures are maintained at 37°C in a water saturated air atmosphere containing 5%CO_2.

Cells are transfected with either the control plasmid (pβactin-16PL vector) or pβactin –ΔpCAR–16PL. In brief, an 80% confluent (approx. $1x10^8$ cells) 15cm dish is

transfected with 15µg plasmid DNA using SuperFect from Qiagen according to the manufacturer's protocol. After 24h, cells are harvested, washed and cell pellet resuspended in 0.5ml Lämmli's buffer. Western blotting supplies are obtained from BioRad unless otherwise stated. Samples are sonicated for 10sec, heat-denatured for 5min at 95°C and cellular debris removed by centrifugation (10min 13krpm Eppendorf). Samples are stored at –20°C until further use. A quantity of 30µl/lane is loaded on to a 12% denaturing polyacrylamide gel (SDS-PAGE) and run at 100V for 90min in 1xTris/Glycine/SDS buffer. Gel is then electrotransfered onto a 0.45µm Protan BA85 (Schleicher&Schuell) nitrocellulose membrane in 1xTris/Glycine buffer (Novex) containing 20% methanol. The membrane is blocked for 1h in phosphate-buffered saline (PBS) containing 5% non-fat dry milk and 1%Tween 20 (Sigma), followed by 1h incubation with an affinity-purified polyclonal chicken-anti human CAR antibody at 1:500 in blocking solution. In between antibody incubation steps the membrane is washed by two short rinses in PBS/1%Tween 20 followed by 2x15min in the same washing buffer. The membrane is incubated for 1h with a biotinylated rabbit-anti chicken IgY (Vector Laboratories) diluted at 1:1000 in blocking solution, followed by 30min incubation with streptavidin-horseradish peroxidase (Vector Laboratories) at 1:1000 in blocking solution. Membrane is incubated for 5min in enhanced chemiluminescence (ECL) substrate (Amersham), solution is carefully drained and membrane put in a Photogene Development folder (Life Technologies). ECL signals are detected by exposing Hyperfilm ECL (Amersham) to the membrane and films are developed on a X-Ray film developer (Agfa).

All 3 different cell lines which are transfected with ΔpCAR–16PL show an additional strong protein band which has the predicted molecular size. As a positive control 100 ng of recombinant human soluble CAR (hCAR) purified from E.coli source is used.

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The polyclonal chicken-anti human CAR antibody used above are prepared as follows: A soluble version of human CAR is generated by PCR using the CAR1 (5'-accggccatggcatatggatttcgccagaa-3' and the CAR2 (5'-accggctcgagagctttatttgaaggagggac-3') primers. As template full length human CAR cloned from HeLa cells is used. The soluble human CAR PCR fragment is digested with Nde1 and Xho1 and inserted into the prokaryotic expression vector pET-17H, which contains a C-terminal histidine tag. The construct is transformed into bacteria and cells are induced to produce the soluble human

CAR protein. The protein is purified by commonly used methods and is injected into an adult female chick. The eggs of the hen are collected and antibodies isolated from the egg yolk.

<u>Example 3:</u> Functionality of ΔpCAR in mammalian cells (adenoviral gene transfer)

The functionality is tested by transient transfection of CHO cells with the construct to be tested or the control plasmid, followed by transduction with an adenovirus which contains a reporter gene.

CHO cells are seeded into 24 well plate at a density of 12'000 cells/well. Cells are transiently transfected with 0.5μg plasmid DNA of either pβactin-16PL or pβactin-ΔpCAR-16PL and incubated for 24h. Cells are then transduced with an adenoviral vector carrying β-galactosidase as a reporter gene (moi 0-100) for 2h. Virus solution is removed and cells incubated for an additional 4 days. Reporter gene expression is monitored using staining for nuclear β-galactosidase. Only ΔpCAR transfected cells are transduced with the reporter gene.

<u>Example 4:</u> Generation of transgenic mice

(a) <u>Generation of ΔpCAR BALB/c ES cell lines</u>

$5x10^6$ BALB/c ES cells ("Efficient targeting of the IL-4 gene in a BALB/c embryonic stem cell line", Noben-Trauth *et al.*, Transgenic-Res. 1996 Nov; 5(6): 487-91) are electroporated with 30 μg of the linearized construct. Transfected cells are selected with G418 (200 μg/ml). G418-resistant clones are screened for integration events by PCR. The ES cells are lysed 1h/37°C with 20 μl Lysis buffer (PCR buffer 1X; SDS 1.7 μM; Proteinase K 50 μg/ml) heat inactivated 85°C/15 Min. and cleared by centrifugation. 1,3 μl lysed solution is used in for a 50 μl PCR. Positive clones are further verified by Southern analysis.

(b) <u>Generation of ΔpCAR transgenic mice</u>

BALB/c-ES cell clones carrying one ΔpCAR allele are injected into C57BL/6 host blastocysts and transferred into pseudopregnant foster mothers according to standard protocols. Chimaeras are mated with BALB/c females and albino offspring (indicative for germ line transmission) are analyzed by PCR for target integration and Southern analysis. Heterozygous animals are generated by back-crossing of F1 animals to Balb/c wild type

animals and Southern analysis of the F2 animals. The homozygous lines are established by mating heterozygous F1 animals.

<u>**Example 5:**</u> Transplantation

Hearts of transgenic mice obtained according to Example 4 are removed, in vitro transduced by infusion with an adenovirus carrying β-galactosidase and then heterotopically transplanted into female mice (which do not express pCAR). Age matched Balb/c male mice are used as controls. 4 days after transplantation hearts are removed, perfusion stained for nuclear β-galactosidase, paraffin embedded and sectioned. Sectiones are counterstained with hematoxylin and evaluated by light microscopy. Positive expression for β-galactosidase is seen in the transgenic mice compared to the control animals.